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**EXHIBIT D**

PAGE 26/34 \* RCVD AT 5/21/2004 5:57:18 PM [Eastern Daylight Time] \* SVR:USPTO-EFXRF-1/0 \* DNIS:8729306 \* CSID:650 496 1200 \* DURATION (mm-ss):12-20

10-fold decline in CD4<sup>+</sup> T cells that is typically seen in the course of AIDS, the frequency of infection in these cells is at least 100-fold greater than in early seropositives. It is this high level of infection that may be the primary cause for the relentless and often accelerated decline in T4 cell number and function in patients with AIDS.

Evidence is accumulating to support the theory that monocytes and macrophages, subsets of which express the CD4 molecule (15), are involved in the pathogenesis of HIV-1 infection. It has been shown that monocytes can be infected *in vitro* with HIV-1, and the virus can be isolated from or identified in monocytes obtained from the blood as well as from cells of monocyte lineage in the brain and alveolar macrophages in the lung (16-18). Using PCR to examine highly enriched sorted monocytes from HIV-1-infected individuals, we have found that only a minority of such individuals have demonstrable HIV-1 in their peripheral monocytes, whereas all have HIV-1 DNA in their CD4<sup>+</sup> T cells. One may theorize that unlike the CD4<sup>+</sup> T cells and tissue macrophages, the relatively immature monocytes found in the peripheral blood are less susceptible to infection by HIV-1.

Having determined that the CD4<sup>+</sup> T cell is the primary cell in the peripheral blood that contains HIV-1, it will be important to determine what portion of these cells contain latent HIV-1 exclusively and to try and identify those properties of HIV-1 that maintain it in a latent state. In this regard, PCR may be used to amplify specific regulatory regions of HIV-1 such as *nef* (3'-orf) and to determine their level of transcriptional activation in different stages of infection in order to study factors that may control latency.

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19. Fluorescently labeled PBMCs were sorted with an EPICS C flow cytometer (Coulter Electronics) equipped with a 2-W argon laser with an excitation wavelength of 488 nm. Sorting logic was controlled by the use of forward angle light scatter versus log green fluorescence bit map gating. Cellular debris, red blood cells, and cell aggregates were eliminated from sorting by forward angle light scatter. The cells reactive with each monoclonal antibody were determined by comparing the fluorescence of cells stained with specific antibody and cells that had been incubated with FITC-labeled isotype controls. Cells were sorted at a rate of approximately 2000 cells per second with coincidence abort engaged. Both positively and negatively stained cells meeting the appropriate sort criterion were collected into separate tubes containing sterile phosphate-buffered saline (PBS). On completion of cell sorting, both positive and negative cell populations were analyzed for purity with machine parameters identical to those for sorting.
20. The RT assay used for these experiments was carried out as previously described [R. L. Willey et al., *J. Virol.* 62, 139 (1988)]. Briefly, 10  $\mu$ l of culture supernatant was added to a cocktail containing polyadenylated oligo(dT) (Pharmacia), MgCl<sub>2</sub>, and <sup>32</sup>P-labeled thymidine triphosphate (Amersham) and was incubated at 37°C for 2 hours. Five microliters of the cocktail was then spotted onto DE81 paper, dried, and washed in 1× saline sodium citrate buffer. The paper was dried and exposed to radiographic film for 12 hours at -80°C.
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23. Cells to be examined for HIV-1 DNA were washed and pelleted in PBS, and lysed in 0.001% Triton X-100/0.0001% SDS in TE buffer (10 mM Tris-HCl, pH 8, and 0.5 mM EDTA, pH 8) with 600  $\mu$ g per milliliter of proteinase K (Boehringer Mannheim) for 1 hour at 56°C, 15 min at 95°C. The PCR reaction mixture contained 50  $\mu$ l of DNA lysate, 50 pmol each of primers, 200  $\mu$ M each of four deoxynucleotide triphosphates (Boehringer Mannheim), 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.2% gelatin, and 2 units of thermostable DNA polymerase from *Thermus aquaticus* (Perkin-Elmer Cetus). Primer pairs used in these experiments included SK 68/69 (mv 7801-7820, 7922-7942), SK38/39 (gag 1551-1578, gag 1665), SK29/30 (LTR 501-518, LTR 589-605), and QH26/27 (HLA-DQ $\alpha$  control) as previously described (6). Amplification was carried out by means of a programmable thermal cycler (Perkin-Elmer Cetus) with denaturation at 94°C, annealing at 55°C, and extension at 72°C for 30 cycles. After amplification, 20  $\mu$ l of each were taken from HLA-DQ $\alpha$  control tubes for agarose gel electrophoresis (3% NuSieve, 1% agarose) to determine if amplification had occurred. Aliquots of 30  $\mu$ l were taken from the test amplification tubes, mixed with a <sup>32</sup>P-labeled adenosine triphosphate (ATP) end-labeled probe (SK70, mv 7841 to 7875; SK19, gag 1595 to 1635; and SK31, LTR 552 to 585 probes for the mv, gag, and LTR primers, respectively) (6), denatured at 94°C, and hybridized at 56°C for 15 min. The hybridized heteroduplexes were then analyzed on a 10% polyacrylamide gel. Autoradiographs of the gels were obtained by exposure of Kodak XAR film at -70°C at 2, 4, and 16 hours in the presence of an intensifying screen.
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## Antibody to Interleukin-5 Inhibits Helminth-Induced Eosinophilia in Mice

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When rodents are infected with the nematode *Nippostrongylus brasiliensis*, large numbers of eosinophils appear in their blood and lungs and their serum immunoglobulin E (IgE) is increased. Injection of a monoclonal antibody to interleukin-5 completely suppressed the blood eosinophilia and the infiltration of eosinophils in the lungs of parasitized mice but had no effect on serum IgE. In contrast, an antibody to interleukin-4 inhibited parasite-induced IgE but not the eosinophilia. These results show that interleukin-5 is important in eosinophil production *in vivo* and that IgE and eosinophil production are regulated by different cytokines produced by the T<sub>H</sub>2 subset of CD4-expressing T cells.

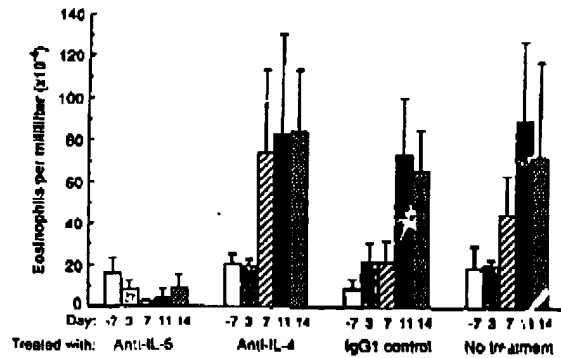
**B**LOOD AND TISSUE EOSINOPHILIA, large immunoglobulin E (IgE) responses, and intestinal mastocytosis are characteristic of the mammalian host response to helminth parasites (1). All three responses appear to be regulated by T lymphocytes since they do not occur in athymic

(2) or T cell-depleted animals (3). Both mouse and human interleukin-5 (IL-5) have been shown to be potent and specific

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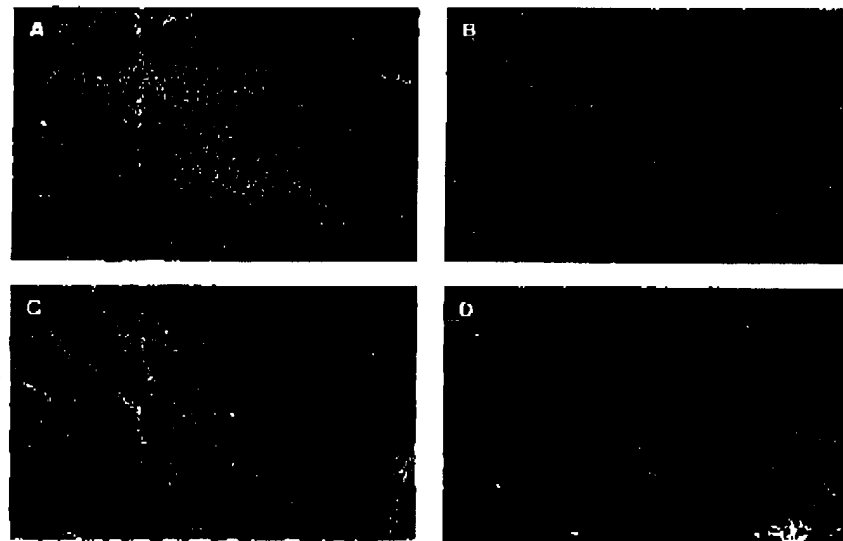
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**Fig. 1.** Inhibition of Nb-induced eosinophilia by anti-IL-5. Twelve-week-old female BALB/c mice were injected subcutaneously with 750 third-stage Nb larvae. On the same day (day 0) some groups of mice received antibodies intraperitoneally. Animals were injected with anti-IL-5 (TRFK-5) (2 mg per mouse), anti-IL-4 (11B11) (10 mg per mouse) or an IgG1 isotype-matched control (antibody GL113 to  $\beta$ -galactosidase) (10 mg per mouse) (16). Animals were bled from the tail vein on the days indicated and the eosinophils were counted in Discombe's fluid on a hemacytometer (17). Data represent the mean ( $\pm$  SD) eosinophil counts per milliliter in groups of five mice.



**Table 1.** Serum IgE levels were determined by an IgE-specific enzyme-linked immunosorbent assay (15). The results are from the same experiment as in Fig. 1. Given are the mean IgE levels in groups of five mice, with the SD in parentheses.

Treatment	Total serum IgE ( $\mu$ g/ml) on days		
	-7	11	14
No antibody	0.87 (0.43)	32.4 (9.7)	31.1 (11.2)
Anti-IL-5	0.53 (0.34)	34.3 (19.9)	34.9 (20)
Anti-IL-4	1.85 (0.83)	2.5 (1.6)	3.5 (2.4)
IgG1 control	1.15 (0.23)	12.0 (6.7)	24.8 (7.7)



**Fig. 2.** Effect of anti-IL-5 on eosinophilic infiltration of lung. (A) Lung of a normal mouse ( $\times 40$ ). (B) Lung removed from a mouse 14 days after injection with Nb showed extensive cellular infiltration ( $\times 40$ ). (C) Lung of a parasitized mouse (same as shown in (B)), viewed at higher magnification ( $\times 400$ ). Cellular infiltrates contained large numbers of eosinophils, which are distinguished from other cells by a segmented nucleus and pink cytoplasmic granules. (D) Lung of a parasitized mouse treated with anti-IL-5 antibody ( $\times 400$ ). Wright-Giemsa stain was used in (A) to (D).

stimuli for eosinophil colony formation in semisolid cultures (4) and for eosinophil growth and differentiation in liquid cultures (5). These findings suggested that IL-5 may be an important mediator of the eosinophilic response to parasites. Rodents infected

with the intestinal nematode *Nippostrongylus brasiliensis* (Nb) have been extensively studied as models of host responses to helminth parasites (6). We assessed the importance of IL-5 for blood and tissue eosinophilia and for the generation of eosinophil precursors

by treating Nb-infected mice with a monoclonal antibody specific for IL-5 (7).

Inoculation of BALB/c mice with third-stage Nb larvae led to a 4- to 8-fold increase in blood eosinophils and a 25- to 100-fold increase in serum IgE levels. Both responses were rapid and reached a maximum by days 11 to 14 (Fig. 1 and Table 1). No increases in eosinophil numbers were observed in animals that received 2 mg of TRFK-5, a monoclonal antibody to IL-5, and in most experiments the number of blood eosinophils decreased from starting levels (Fig. 1). A monoclonal antibody to IL-4 (anti-IL-4) (8) and an isotype-matched control did not significantly reduce the number of circulating eosinophils. Injection of antibody to IL-5 (anti-IL-5) at 2 mg per mouse consistently caused complete inhibition of eosinophilia, whereas injection at 0.25 mg per mouse caused substantial, but not total, inhibition (9). Total white blood cell counts increased less than twofold and was not correlated with either of the antibody treatments (9).

In contrast to the above result, anti-IL-4, but not anti-IL-5, caused almost total inhibition of the parasite-induced elevation of IgE (Table 1). The inhibition of Nb-induced IgE by anti-IL-4 was reported earlier (10), and our purpose here was to show that the lack of effect of anti-IL-4 on eosinophilia was not due to insufficient antibody. The inability of anti-IL-5 to inhibit the IgE response is consistent with experiments *in vitro* which show that the induction of IgE by T<sub>H</sub>2 clones (which produce both IL-4 and IL-5) (11) can be inhibited by anti-IL-4 but not by anti-IL-5 (12).

The effect of anti-IL-5 on eosinophil infiltration into the lung was similar to that on blood eosinophilia. *Nippostrongylus brasiliensis* larvae pass through and molt in the lung during the first few days of infection. Histological examination of the lung 14 days after challenge with Nb revealed numerous localized cellular infiltrates not seen in normal lung (Fig. 2, A and B). These infiltrates appeared as focal aggregates of monocytes, neutrophilic granulocytes, and eosinophils (Fig. 2C). Lungs of parasitized mice treated with anti-IL-5, anti-IL-4, or an isotype control were also highly infiltrated. However, the foci formed in the animals treated with anti-IL-5 were virtually devoid of eosinophils (Fig. 2D). To quantitate these impressions, the average number of eosinophils per high-power field were counted in two lung sections from each of four mice. The results (mean  $\pm$  SD) were  $0.5 \pm 1.2$  for anti-IL-5;  $59 \pm 25$  for anti-IL-4;  $48 \pm 15$  for the isotype control; and  $63 \pm 31$  when no antibody was used. None of the antibodies appeared to change the total number of localized infiltrates observed

or their monocyte and granulocyte content. Thus, IL-5 appears to be as essential for tissue eosinophilia as for blood eosinophilia.

We proposed earlier that T cell-dependent elevations of IgE and eosinophils that typically accompany both atopic diseases and helminth parasite infections are regulated by a combination of cytokines produced only by the  $T_H2$  subset of mouse CD4-expressing T cells (13). On the basis of activities observed in vitro, IL-4 was predicted to be the key factor responsible for the increase in IgE and IL-5 for the generation of eosinophils. Our experiments show that IL-5 is essential in the eosinophilia induced by Nb and confirm that IL-4 is required for the IgE response to Nb (10). Thus, different secreted products of  $T_H2$  cells appear to regulate both the production of IgE and one of the cell types that uses IgE to confer specificity on its effector functions.

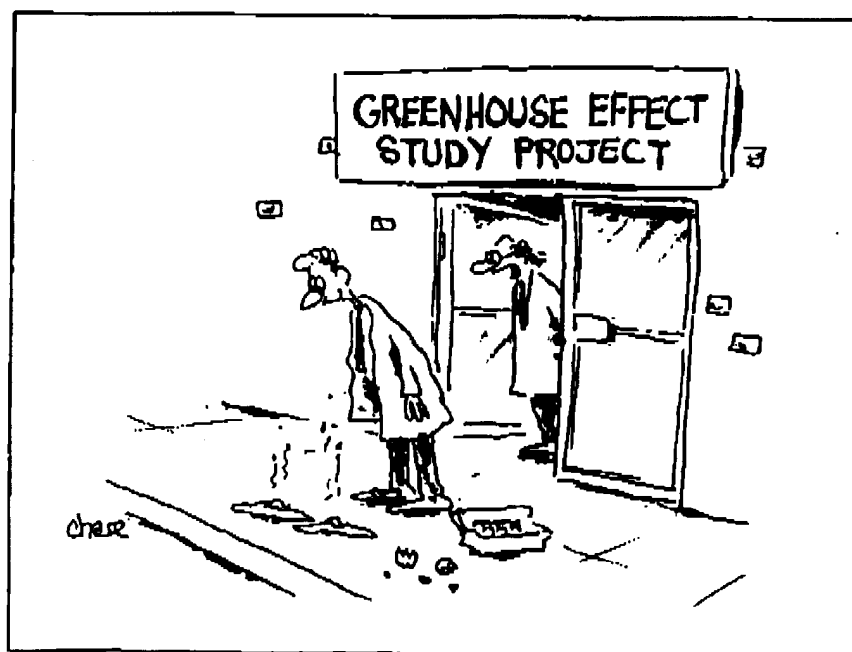
Our results have been confirmed in mice infected with *Schistosoma mansoni*, in which both blood eosinophilia and eosinophil infiltration of hepatic granulomas were inhibited by the antibody TRFK-5 (14). Both the

extent of lung infiltration in Nb-infected mice and the number and size of hepatic granulomas in *S. mansoni*-infected mice were comparable in the groups treated with anti-IL-5 and with control antibody. The only difference in each case was that eosinophils were virtually absent after treatment with anti-IL-5. These findings suggest that agents that specifically inhibit the synthesis or function of IL-5 may be useful in diseases in which eosinophil infiltration is prominent, such as bronchial asthma, tropical pulmonary eosinophilia, or hypereosinophilic syndrome.

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"Simpkins, I wish you'd take this study more seriously!"